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(19) (CA) **CANADIAN PATENT** (12)

(54) Methods and Structures Employing Non-Radioactive Chemically-Labeled Polynucleotide Probes

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(30) (US) U.S.A. 461,469 1983/01/27

(57) 28 Claims

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ABSTRACT OF THE DISCLOSURE

Non-radioactive chemically-labeled polynucleotide probes, such as non-radioactive chemically-labeled, single-stranded DNA probes, are hybridized to fixed, denatured, single-stranded genetic material having a make-up complementary to said probes for determining the identification and/or presence of said genetic material. The non-radioactive chemical label on said probe is employed to elicit a response therefrom so as to determine the identity and/or presence of said genetic material. It is preferred that the non-radioactive chemical label attached to the probe have an enzyme attached thereto after hybridization, and that the presence of the enzyme attached to the chemically-labeled probe be elicited by spectrophotometric assay techniques.

THE EMBODIMENTS OF THE PRESENT INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS DEFINED ARE CLAIMED AS FOLLOWS:

1. A method for detecting a polynucleotide sequence which comprises:

fixing said polynucleotide sequence to a solid support which comprises or is contained within a transparent or translucent, non-porous system, such that a single-strand of the polynucleotide is capable of hybridizing to complementary nucleic acid sequences;

forming an entity comprising said polynucleotide sequence hybridized to a polynucleotide or oligonucleotide probe, said probe having attached thereto a chemical label further comprising a signalling moiety capable of generating a soluble signal; and

generating and detecting said soluble signal.

2. The method according to claim 1, wherein said detecting step comprises spectrophotometric techniques.

3. The method according to claim 1, wherein said soluble signal is selected from the group consisting of a colored product, a chemiluminescent product and a fluorescent product.

4. The method according to claim 1, wherein said signalling moiety is selected from the group consisting of an enzyme, a chelating agent and a co-enzyme.

5. The method according to claim 1, wherein said solid support is selected from the group consisting of glass, plastic, polystyrene, polyethylene, dextran and polypropylene.

6. The method according to claim 1, wherein said polynucleotide sequence is directly fixed to said solid support.

7. The method according to claim 6, wherein said polynucleotide sequence is fixed to said solid support in single stranded form.

8. The method according to claim 1, wherein said signalling moiety is attached to said polynucleotide or oligonucleotide probe through the formation of a complex.

9. The method according to claim 8, wherein said complex is selected from the group consisting of biotin and avidin, biotin and streptavidin, and a sugar and a lectin.

10. The method according to claim 1, wherein said forming step further comprises washing to remove said polynucleotide or oligonucleotide probes that do not form said entity.

11. The method according to claim 10, wherein said forming step further comprises attaching said signalling moiety to said polynucleotide or oligonucleotide probe after said washing step.

12. The method according to claim 11, which further comprises separating free signalling moieties from said attached signalling moieties.

13. The method according to claim 1, wherein said detecting step further comprises generating said soluble signal in a device capable of transmitting light therethrough for the detection of said soluble signal by spectrophotometric techniques.

14. The method according to claim 13, wherein said device is selected from the group consisting of a well, a tube, a cuvette and an apparatus which comprises a plurality of said wells, tubes or cuvettes.

15. The method according to claim 13, wherein said soluble signal is selected from the group consisting of a colored product, a chemiluminescent product, and a fluorescent product.

16. The method according to claim 13, wherein said solid support and said device are composed of the same materials.

17. A kit for detecting a polynucleotide sequence comprising a device, said device comprising a solid support and having said polynucleotide sequence fixed thereto in hybridizable form, in packaged combination with a container of an oligonucleotide or polynucleotide probe, having covalently attached thereto a chemical label comprising a signalling moiety capable of generating a soluble signal.

18. The kit of claim 17, wherein said soluble signal is a colored product or a fluorescent product.

19. The method according to claim 1 wherein part of the solid support is modified to facilitate fixing of the polynucleotide sequence to the solid support by the sequential steps of:

(a) heating or boiling the solid support in dilute nitric acid for about 45 minutes;

(b) washing or rinsing the solid support with distilled water;

(d) drying the solid support at about 115°C, for about 24 hours;

(d) incubating the solid support in contact with 10% gamma-aminopropyltriethoxysilane for about two to three hours at about 45°C;

(e) washing with water; and

(f) drying overnight at a temperature of about 100°C.

20. The method according to claim 1 wherein part of the solid support is modified to facilitate fixation of the polynucleotide sequence to the solid support by treating the solid support with a coating of an epoxy resin.

21. The method according to claim 20 wherein part of the solid support is modified to facilitate fixation of the polynucleotide sequence to the solid support by treating the support with the epoxy resin by the following sequential steps;

(a) applying an epoxy glue in solution with ethanol to the solid support; and

(b) evaporating the ethanol by heating to a temperature of about 37°C to provide a polyamine polymeric coating on the solid support.

22. The method according to claim 1 wherein said polynucleotide sequence is fixed to said solid support in double-stranded form, and denatured into single-stranded form prior to the hybridization step.

23. The method according to claim 1 wherein said polynucleotide sequence in double-stranded form is denatured,

said fixed to said solid support in single-stranded form prior to the hybridization step.

24. The method according to claim 1 wherein said polynucleotide sequence to be detected is in single-stranded form and is indirectly bound to said solid support by sandwich hybridization.

25. The method according to claim 1 wherein a cell or cellular material is directly fixed to said solid support, and polynucleotide sequences within said material are hybridized to polynucleotide or oligonucleotide probes in situ.

26. The method according to claim 1 wherein said signalling moiety is a chemiluminescent agent.

27. A composition of matter which comprises a transparent or translucent non-porous system further comprising a double-stranded polynucleotide which is immobilized on a solid support wherein one of the strands is chemically labelled and capable of generating a soluble signal.

28. The composition of claim 27 wherein the solid support is contained within the transparent or translucent non-porous system.

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BACKGROUND OF THE DISCLOSURE

In the determination of the presence or the identity of genetic material, such as DNA genetic material, it has been proposed to denature the genetic material to form single-stranded DNA or single-stranded genetic material. The single-stranded genetic material is then fixed to a solid support and contacted with a probe, such as a DNA probe, having in its make up bases complementary to the make up of the fixed genetic material to be identified and/or determined. The contacting of the single-stranded genetic material along with the single-stranded probe is carried out under conditions to effect hybridization of the genetic material to be determined or identified and the probe.

Radioactively-labeled probes, such as radioactively-labeled single-stranded DNA probes, have been employed. U.S. Patent 4,358,535 discloses a methcd of identifying a pathogen present in the clinical sample by denaturing the genetic material present in the clinical sample to form single-stranded genetic material thereof and to fix the resulting single-stranded genetic material characterizing the pathogen to an inert support or surface. The thus-fixed single-stranded genetic material characterizing or identifying the pathogen is brought into contact with a radioactive single-stranded probe under hybridizing conditions to effect duplex form or double-strand formation of the genetic material derived from the pathogen and the probe. The presence of the resulting formed duplex between the probe and the pathogen genetic material would then be detected and would confirm the presence and/or identity of the pathogen.

The disadvantages of employing a radioactively-labeled probe, such as a radioactively-labeled DNA probe, for the identification of genetic material are well known to those skilled in the art. Such disadvantages include not only the precautions and hazards involved in handling the radioactive material but also the short life of such radioactive material and expense in connection with the handling and use of such radioactively-labeled DNA probes.

It is known to chemically-label nucleotides and polynucleotides to avoid the hazards and/or difficulties associated when such compounds are radioactively-labeled. For example in the article by P.R. Langer, A.A. Waldrop and D.C. Ward entitled "Enzymatic Synthesis of Biotin-Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes", in Proc. Natl. Acad. Sci., USA, Vol. 78, No. 11, pp. 6633-6637, November 1981, there are described analogs of dUTP and UTP that contain a biotin molecule bound to the C-5 position of the pyrimidine ring through an allylamine linker arm. The biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantitatively retained on avidin-Sepharose,* even after extensive washing with 8M urea, 6M guanidine hydrochloride or 99% formamide. In addition, biotin-labeled nucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and Staphylococcus aurea, Protein A. These unique features of biotin-labeled polynucleotides suggest that they are useful affinity probes for the detection and isolation of specific DNA and RNA sequences. It is

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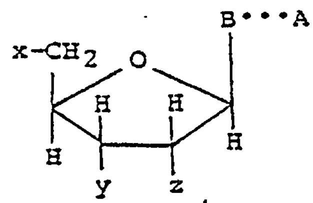
* Sepharose is a trademark

indicated in the article that the subject matter of the article is comprised in a pending U.S. patent application.

Compounds or nucleotides have also been prepared which can be incorporated into DNA, such as double-stranded DNA, and which are useful for the preparation of non-radioactive chemically-labeled DNA probes. See, for example, European Patent Application S.N. 0063879, filed 6 April, 1982, and published 3 November, 1982, in which the subject matter of the above-identified article is disclosed, and additionally it is disclosed that compounds having the structure:



wherein B represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 9-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

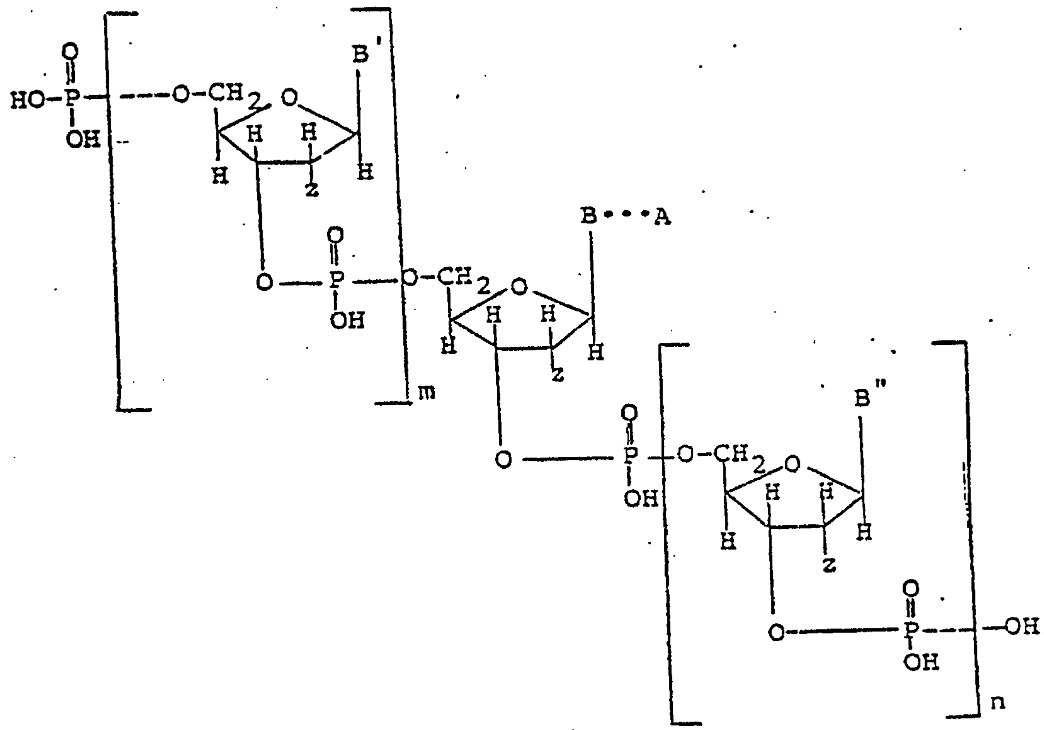
wherein each of x, y and z represents

$$H-,\ HO-,\ HO-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-,\ HO-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-,\ \text{or}\ HO-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-\overset{O}{\underset{OH}{\overset{\|}{\underset{|}{P}}}}-O-,$$

are widely useful as probes in biomedical research and recombinant DNA technology.

Particularly useful are compounds encompassed within this structure which additionally have one or more of the following characteristics: A is non-aromatic; A is at least C_5; the chemical linkage joining B and A includes an α-olefinic bond; A is biotin or iminobiotin; and B is a pyrimidine or 7-deazapurine.

European Patent Application Serial No. 0063879 also discloses compounds having the structure:



wherein each of B, B', and B" represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that whenever B, B', or B" is purine or 7-deazapurine, it is attached at the N^9-position of the purine or 7-deazapurine, and whenever B, B', or B" is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded duplex formed with a complementary ribonucleic or deoxyribonucleic acid molecule;

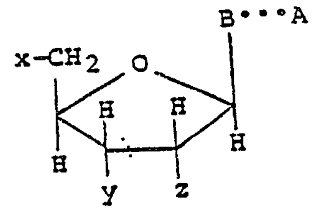
wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 8-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine;

wherein z represents H- or HO-; and

wherein m and n represent integers from 0 up to about 100,000.

These compounds can be prepared by enzymatic polymerization of a mixture of nucleotides which include the modified nucleotides of this invention. Alternatively, nucleotides present in oligo- or polynucleotides may be modified using chemical methods.

The chemically-labeled or modified nucleotides described in the above-referred PNAS article and in European Patent Application Serial No. 0063879 as indicated hereinabove is the structure:

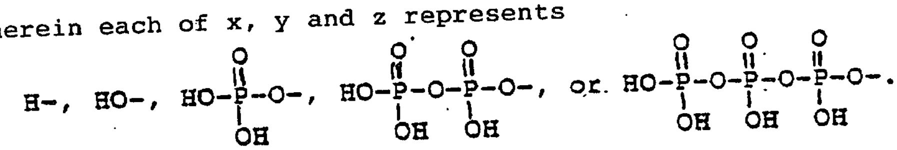


wherein B represents a purine, 7-deazapurine, or pyrimidine moiety covalently bonded to the C^1-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^9-position of the purine or 7-deazapurine, and when B is pyrimidine, it is attached at the N^1-position;

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid;

wherein the dotted line represents a linkage group joining B and A, provided that if B is purine, the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y and z represents



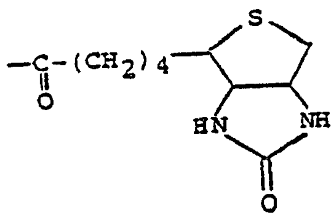
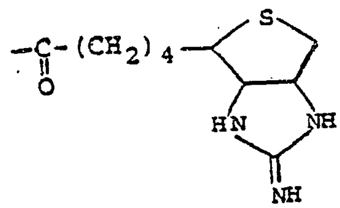
These compounds are widely useful as probes in biomedical research and recombinant DNA technology.

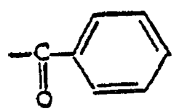
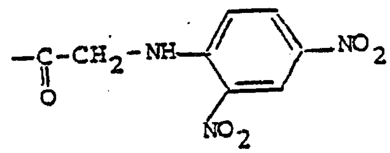
Although in principal, all compounds encompassed within this structural formula may be prepared and used in accordance with the practices of this invention, certain of the compounds are more readily prepared or used or both, and therefore are presently preferred.

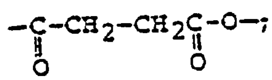
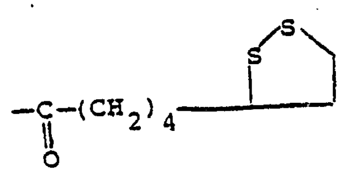
Thus, although purines, pyrimidines and 7-deazapurines are in principal useful, pyrimidines and 7-deazapurines are preferred since purine substitution at the 8-position tends to render the nucleotides ineffective as polymerase substrates. Thus, although modified purines are useful in certain respects, they are not as generally useful as pyrimidines and 7-deazapurines. Moreover, pyrimidines and 7-deazapurines useful in this invention must not be naturally substituted at the 5- or 7- positions, respectively. As a result, certain bases, such as thymine, 5-methylcytosine, and 5-hydroxymethylcytosine, are not useful. Presently preferred bases are cytosine, uracil, deazaadenine and deazaguanine.

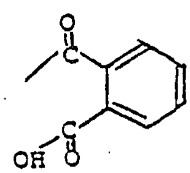
A may be any moiety which has at least three carbon atoms and is capable of forming a detectable complex with a polypeptide when the modified nucleotide is incorporated into a double-stranded duplex containing either deoxyribonucleic or ribonucleic aicd.

A therefore may be any ligand which possesses these properties, including haptens which are only immunogenic when attached to a suitable carrier, but are capable of interacting with appropriate antibodies to produce complexes. Examples of moieties which are useful include:

 ;  ;

 ;  ;

 ;  ; and



Of these, the preferred A moieties are biotin and iminobiotin.

Moreover, since aromatic moieties tend to intercalate into a base-paired helical structure, it is preferred that the moiety A be nonaromatic. Also, since smaller moieties may not permit sufficient molecular interaction with polypeptides, it is preferred that A be at least C_5 so that sufficient interaction can occur to permit formation of stable complexes. Biotin and iminobiotin satisfy both of these criteria.

The linkage or group joining moiety A to base B may include any of the well known bonds, including carbon-carbon single bonds, carbon-carbon double bonds, carbon-nitrogen single bonds, or carbon-oxygen single bonds. However, it is generally preferred that the chemical linkage include an olefinic bond at the α-position relative to B. The presence of such an α-olefinic bond serves to hold the moiety A away from the base when the base is paired with another in the well known double-helix configuration. This permits interaction with polypeptide to occur more readily, thereby facilitating complex formation. Moreover, single bonds with greater rotational freedom may not always hold the moiety sufficiently apart from the helix to permit recognition by and complex formation with polypeptide.

It is even more preferred that the chemical linkage group be derived from a primary amine, and have the structure $-CH_2-NH-$, since such linkages are easily formed utilizing any of the well known amine modification reactions. Examples of preferred linkages derived from allylamine and allyl-(3-amino-2-hydroxy-1-propyl) ether groups have the formulae $-CH=CH-CH_2-NH-$ and $-CH=CH-CH_2-O-CH_2-\underset{\displaystyle OH}{\underset{|}{C}H}-CH_2-NH-$,

respectively.

Although these linkages are preferred, others can be used, including particularly olefin linkage arms with other modifiable functionalities such as thiol, carboxylic acid, and epoxide functionalities.

The linkage groups are attached at specific positions, namely, the 5-position of a pyrimidine, the 8-position of a purine, or the 7-position of a deazapurine. As indicated previously, substitution at the 8-position of a purine does not produce a modified nucleotide which is useful in all the methods discussed herein. It may be that the 7-position of a purine, which is occupied by a nitrogen atom, could be the point of linkage attachment. However, the chemical substitution methods employed to date and discussed herein are not suitable for this purpose.

The letters x, y, and z represent groups attached to the 5', 3' and 2' positions of the sugar moiety. They may be any of

$$\mathrm{H{-}},\quad \mathrm{HO{-}},\quad \mathrm{HO{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}},\quad \mathrm{HO{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}},\quad \text{or}\quad \mathrm{HO{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}}\underset{\mathrm{OH}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}\mathrm{{-}O{-}}.$$

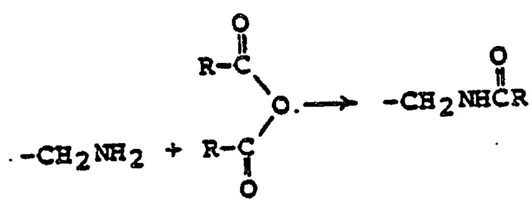
Although conceivable, it is unlikely that all of x, y, and z will simultaneously be the same. More likely, at least one of x, y, and z will be a phosphate-containing group, either mono-, di-, or tri-phosphate, and at least one will be HO- or H-. As will be readily appreciated, the most likely identity of z will be HO- or H- indicating ribonucleotide or deoxyribonucleotide, respectively. Examples of such nucleotides include 5'-ribonucleoside monophosphates, 5'-ribonucleoside diphosphates, 5'-deoxyribonucleoside triphosphates, 5'p-ribonucleoside-3'p, and 5'p-deoxyribonucleoside-3'p. More specific examples include modified nucleotides of this type in which A is biotin or iminobiotin, the chemical linkage is

$$-CH{=}CH-CH_2-NH- \text{ or } -CH{=}CH-CH_2-O-CH_2-\underset{OH}{\underset{|}{C}H}-CH_2-NH-,$$

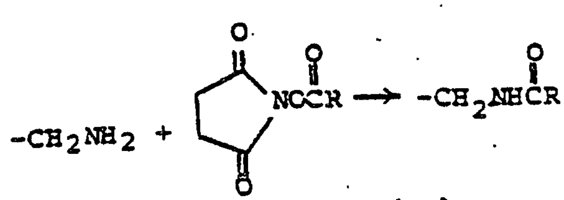
and B is uracil or cytosine.

The general synthetic approach adopted for introducing the linker arm and probe moiety onto the base is discussed thereinabove. (See especially, D.E. Bergstrom, and M.K. Ogawa, J. Amer. Chem. Soc. <u>100</u>, 8106, 1978; and C.F. Bigge, P. Kalaritis, J.R. Deck and M.P. Mertes, J. Amer. Chem. Soc. <u>102</u>, 2033, 1980.) However, the olefin substituents employed herein have not been used previously. To facilitate attachment of probe moiety A, it has been found particularly desirable to employ olefins with primary amine functional groups, such as allylamine [AA] or allyl-(3-amino-2-hydroxy-1-propyl) ether [NAGE], which permit probe attachment by standard amine modification reactions, such as,

$$-CH_2NH_2 + \underset{\text{Imidate}}{R-\overset{\overset{+}{N}H_2}{\overset{\|}{C}}-OR} \longrightarrow -CH_2NH\overset{\overset{+}{N}H_2}{\overset{\|}{C}}R$$



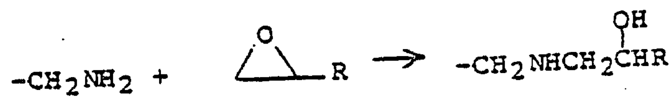
Anhydride



NHS-ester (N-hydroxysuccinimide)

$$-CH_2NH_2 + R-N{=}C{=}S \longrightarrow -CH_2NH\overset{\overset{\displaystyle S}{\|}}{C}NHR$$

Isothiocyanate



Epoxide

Because of ease of preparation, it has been found preferable to use NHS-esters for probe addition. However, olefin linker arms with other modifiable functional groups, such as thiols, carboxylic acids, epoxides, and the like, can also be employed. Furthermore, both linker arm and probe can be added in a single-step if deemed desirable.

Having the biotin probe directly attached to the nucleotide derivatives that are capable of functioning as enzyme substrates offers considerable versatility, both in the experimental protocols that can be performed and in the detection methods (microscopic and non-microscopic) that can be utilized for analysis. For example, biotin nucleotides can be introduced into polynucleotides which are in the process of being synthesized by cells or crude cell extracts, thus making it possible to detect and/or isolate nascent (growing) polynucleotide chains. Such a procedure is impossible to do by any direct chemical modification methcd. Furthermore, enzymes can be used as reagents for introducing probes such as biotin into highly selective or site-specific locations in polynucleotides; the chemical synthesis of similar probe-modified products would be extremely difficult to achieve at best.

Further, there is disclosed in copending, Canadian patent application Serial No. 430,882, filed 21 June, 1983. modified non-radioactive chemically-labeled nucleotides wherein the nucleotides modified such as at the 5-position of pyrimidine or the 7-position of purine, preparatory for the preparation of nucleotide probes therefrom suitable for attachment to or incorporation into DNA or other nucleic acid materials. In the preparation of such modified nucleotides, the nucleotides, i.e. nucleic acids, preferably are modified in a non-disruptive manner such that the resulting modified nuclectides are capable of incorporation into nucleic acids and once incorporated in nucleic acids, the modified nucleotides do not significantly interfere with the formation or stabilization of the double helix formed of the resulting nucleic acids containing the modified nucleotides. The non-disruptive modification of nucleotides and nucleic acids incorporating such modified nucleotides is in contrast with those modifications of nucleotides which are characterized as a disruptive modification in the sense that the resulting disruptively modified nucleotides and nucleic acids containing the same block proper double helix formation. In the practices of this invention, the nucleotides are desirably modified at the 5-position of the pyrimidine or the 7-position of the purine. The nucleotides so modified are non-disruptively modified and nucleic acids containing such nucleotides are capable of forming a double helix arrangement.

Broadly, in another aspect of the practices of this invention, various methods are useful for the tagging or labeling of DNA in a non-disruptive manner. For example, biotin is added on the end of a DNA or RNA molecule. The addition of biotin is accomplished by addition of a ribonucleotide. The 3',4' vicinal hydroxyl groups are oxidized by periodate oxidation and then reduced by a

borohydride in the presence of biotin hydrazide. Alternatively, carbodiimide can also be used to couple biotin to the aldehyde group.

Another technique for tagging nucleic acid material such as DNA or RNA involves the addition of a large marker to the end of a DNA or RNA molecule. One example of this technique is the addition of a molecule, e.g. lysylglycine, where the amino groups are tagged with biotin. Another example would be to follow the procedure set forth hereinabove but employing carbodiimide as the cross-linking agent. Still another example of this techhique would be to produce a biotinylated dA:dU double helical polymer and to ligate this polymer to the probe prepared in accordance with this invention.

Another technique for tagging DNA in a non-disruptive manner involves the isolation of dPyrTP having a putricine or spermidine on the 5-position from PS16 or phage-infected cells. If desired, dPyrTP is made from phage DNA and phosphorylated to dPyrTP followed by modification of the polyamine side chain by means of standard nucleophilic reagent NHS-biotin.

Another technique for tagging DNA in a non-disruptive manner involves the addition of glucose to 5-hydroxymethylcytosine (5 HMC) in DNA using T4 phage glycosylating enzymes followed by screening by means of a lectin-based assay.

Still another method for tagging DNA in a non-disruptive manner involves 5-HMC-triphosphate made from the hydrolysis of T4-DNA followed by phosphorylation of the 5HMCMP to 5 MMCTP. 5 HMCTP is then incorporated into DNA using polymerase I. Thus, any DNA can be modified to have non-disruptively incorporated therein 5 HMC.

A method for tagging DNA in a mildly disruptive manner involves reacting nucleic acids in the double helical form with alkylating reagents as for example benz(o)pyrene diol epoxide or aflatoxin. Under appropriate conditions of the N^2 group of guanine, the N^4 group of adenosine or the N^4 group of cytosine are alkylated. These modified nucleotides can be used as linking arms for the addition of a reporter molecule such as biotin.

These specially modified nucleotides suitable as non-radioactive chemical labels for DNA probes or DNA material as described in Canadian patent application S.N. 430,882, can be broadly characterized and described as phosphoric acid P moiety, a sugar or monosaccharide S moiety, a base B moiety, a purine or a pyrimidine and a signalling chemical moiety Sig covalently attached thereto, either to the P, S or B moiety.

Of special interest are those nucleotides having a general formula

P - S - B - Sig

wherein P is the phosphoric acid moiety including mono-, di-, tri-, or tetraphosphate, S the sugar or monosaccharide moiety, B the base moiety, either a purine or a pyrimidine. The phosphoric acid moiety P is attached at the 3' and/or the 5' position of the S moiety when the nucleotide is a deoxyribonucleotide and at the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B moiety is attached from the N1 position of the N9 position to the 1' position of the S moiety when the base moiety is a pyrimidine or a purine, respectively. The Sig moiety is covalently attached to the B moiety of the nucleotide and when so attached is capable of signalling itself or makes itself self-detecting or its presence known and desirably or preferably permits the incorporation of the resulting nucleotide P - S - B - Sig into or to form a double-stranded helical DNA or RNA or DNA-RNA hybrid and/or to be detectable thereon.

Another special nucleotide in accordance with this invention is characterized by the general formula:

$$\begin{array}{c} \text{Sig} \\ | \\ \text{P} - \text{S} - \text{B} \end{array}$$

Such nucleotides in accordance with this invention would be characterized as ribonucleotides. The phosphoric acid moiety is attached at the 2', 3' and/or 5' position of the sugar S moiety and the base B being attached from the N1 position or the N9 position to the 1' position of the sugar S moiety when said base is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the sugar S moiety is capable of signalling itself or making itself self-detecting or its presence known and preferably permits the incorporation of the ribonucleotide into its corresponding double-stranded RNA or a DNA-RNA hybrid.

Such nucleotides $\begin{array}{c} \text{Sig} \\ | \\ \text{P} - \text{S} - \text{B} \end{array}$ desirably have the Sig chemical moiety attached to the C2' position of the S moiety or the C3' position of the S moiety.

Still further, nucleotides in accordance with the practices of this invention include the nucleotides having the formula

$$\begin{array}{c} \text{Sig} \\ | \\ \text{P} - \text{S} - \text{B} \end{array}$$

wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety. In these special nucleotides, the P moiety is attached to the 3' and/or the 5' position of the S moiety when the nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when

the nucleotide is a ribonucleotide. The base B is either a purine or a pyrimidine and the B moiety is attached from the N1 or the N9 position to the 1' position of the sugar moiety when said B moiety is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the chemical linkage

$$\begin{array}{l} \quad OH \\ \quad\ | \\ -\ P - O - Sig \\ \quad\ \| \\ \quad\ O \end{array},$$

said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded polynucleotide, such as DNA, RNA or DNA-RNA hybrid and when so incorporated therein is still self-detecting.

It is pointed out that the special nucleotides in accordance with the practices of this invention described or defined hereinabove by the general formula P - S - B - Sig, also include nucleotides wherein the Sig chemical moiety is covalently attached to the B moiety at the N^6 or 6-amino group position when the B moiety is adenine or the N^2 or 2-amino group position when the B moiety is guanine or the N^4 or 4-amino group position when the B moiety is cytosine. The resulting nucleotides containing the Sig moiety attached thereto are capable of signalling themselves or making themselves self-detecting or their presence known and being detectable is a double-stranded or DNA, RNA or DNA-RNA hybrid.

By way of summary, as indicated hereinabove with respect to the make up of the various special nucleotides in accordance with this invention, the special nucleotides can be described as comprising a phosphoric acid moiety P, a sugar moiety S and a base moiety B, a purine or pyrimidine, which combination of P-S-B is well known with respect to and defines nucleotides, both deoxyribonucleotides and ribonucleotides. The nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig. The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide, such as a double-stranded DNA, a double-stranded RNA or a double-stranded DNA-RNA hybrid. The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide in accordance with this invention and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation.

The Sig moiety employed in the make up of the special nucleotides of this invention could comprise an enzyme or enzymatic material, such as alkaline phosphatase, glucose oxidase, horseradish peroxidase or ribonuclease. The Sig moiety could also contain a fluorescing component, such as fluorescein or rhodamine or dansyl. If desired, the Sig moiety could include a magnetic component associated or attached thereto, such as a magnetic oxide or magnetic iron oxide, which would

make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an electron dense component, such as ferritin, so as to be available by observation. The Sig moiety could also include a radioactive isotope component, such as radioactive cobalt, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a hapten component or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a polysaccharide or oligosaccharide or monosaccharide, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a chemiluminescent component.

As indicated in accordance with the practices of this invention, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as to the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

The Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in the above-identified European Patent Application SN. 0063879. More specifically, the chemical moiety A described in European Patent Application Serial No. 0063879 is functionally the equivalent of the Sig component or chemical moiety of the special

nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm as described in European patent application No. 0063879, as indicated by the dotted line connecting B and A of the nucleotides therein. The various linker arms or linkages identified in European application 0063879 are applicable to and useful in the preparation of the special nucleotides of this invention.

A particularly important and useful aspect of the special nucleotides of this invention is the use of such nucleotides in the preparation of DNA or RNA probes. Such probes would contain a nucleotide sequence substantially matching the DNA or RNA sequence of genetic material to be located and/or identified. The probe would contain one or more of the special nucleotides of this invention. A probe having a desired nucleotide sequence, such as a single-stranded polynucleotide, either DNA or RNA probe, would then be brought into contact with DNA or RNA genetic material to be identified. Upon the localization of the probe and the formation of a double-stranded polynucleotide containing the probe and the matching DNA or RNA material to be identified, the resulting formed double-stranded DNA or RNA-containing material would then be observable and identified. A probe in accordance with this invention may contain substantially any number of nucleotide units, from about 5 nucleotides up to about 500 or more, as may be required. It would appear that 12 matching, preferably consecutive, nucleotide units would be sufficient to effect an identification of most of the DNA or RNA material to be investigated or identified, if the 12 nucleotide sequence of the probe matches a corresponding cooperative sequence in the DNA

or RNA material being investigated or to be identified. As indicated, such probes may contain one or more of the special Sig-containing nucleotides in accordance with this invention, preferably at least about one special nucleotide per 5-10 of the nucleotides in the probe.

SUMMARY OF THE INVENTION

The invention provides for a method of detecting a polynucleotide sequence. The method first comprises fixing the polynucleotide sequence to a solid support. The support comprises or is contained within a transparent or translucent, non-porous system. A single-strand of the polynucleotide is capable of hybridizing to complementary nucleic acid sequences. The method next comprises forming an entity comprising the polynucleotide sequence hybridized to a polynucleotide or oligonucleotide probe. The probe has attached to it a chemical label. The label comprises a signalling moiety capable of generating a soluble signal. The method further comprises generating and detecting the soluble signal.

In another aspect, the invention provides for a kit for detecting a polynucleotide sequence. The kit comprises a device, being a solid support and having the polynucleotide sequence fixed thereto in hybridizable form, in a packaged combination with a container of an oligonucleotide or polynucleotide probe. The probe has covalently attached thereto a chemical label comprising a signalling moiety capable of generating a soluble signal.

In yet another aspect there is provided a composition of matter which comprises a transparent or translucent non-porous system further comprising a double-stranded polynucleotide which is

double space

immobilized on a solid support wherein one of the strands is chemically labelled and capable of generating a soluble signal. Thus, non-radioactive chemically labelled polynucleotides are prepared such as non-radioactive chemically labelled single-stranded DNA probes. Such probes are then brought into contact with genetic material to be identified or otherwise detected. Before these probes are brought into contact with the genetic material to be identified or investigated, the genetic material is denatured to produce or form single-stranded genetic material therefrom, such as single-stranded DNA derived from the genetic material. Desirably, the resulting single-stranded genetic material is fixed to a suitable inert support or surface such as a plastic material, e.g. polystyrene, preferably a transparent or translucent surface such as glass. Thereupon, the special non-radioactive chemically-labelled probes, such as the special non-radioactive chemically-labelled single-stranded DNA probes of this invention are brought into contact with the thus-fixed single-stranded genetic material under hybridizing conditions. The probe is

selected to provide sufficient number, e.g. at least about 25 bases, in its make up, complementary to the bases making up the genetic material to be detected or identified. The hybridization of the probe to the singlestranded genetic material to be identified with resulting double-stranded or duplex formation would be then detected by means of the non-radioactive chemical label attached to the probe portion of the resulting formed doublestranded hybrid or duplex hybrid. Various techniques, depending upon the non-radioactive chemical label employed in the make up of the probe, may be employed to detect the formation of the double strand or duplex hybrid. It is preferred, however, in the practices of this invention, to employ spectrophotometric techniques and/or enzyme linked immunosorbent assay (ELISA) techniques for the determination of the formed hybrid. Spectrophotometric and ELISA techniques permit not only the detection of the resulting formed double-stranded hybrid, but also permit the quantitative determination thereof, such as by the enzymatic generation of a product that can be measured colorimetrically or fluorometrically. In colorimetric determination, an enzyme linked antibody, such as alkaline phosphatase linked antibody, would be attached to the non-radioactive chemically-labeled probe and employed for the enzymatic generation of the product that can be measured colorimetrically or spectrophotometrically. Another suitable spectrophotometric or colorimetric technique involves immunofluorescence, wherein fluorescein-labeled antibody is employed in the make up prior to or is attached to the non-radioactive chemical label after hybrid formation and the fluorescein-labeled antibody attached or fixed to the probe measured fluorometrically.

Spectrophotometric or colorimetric techniques, in addition to providing the above-identified quantitative result, also usefully provide a fairly prompt visual manifestation or elicitation of the non-radioactive chemical label in the resulting formed double-stranded hybrid. Other ELISA-like or related

techniques are also useful for the detection of the non-radioactive chemical label in the formed duplex, such other techniques would also involve the use of enzymes, e.g. immunoperoxidase, or the use of electron dense markers, e.g. immunoferritin, or other chemical and/or physical markers, attachable or attached to the probes. Broadly, the practices of this invention provide techniques comparable to enzyme linked immunosorbent assay techniques, not only for the qualitative, but also the quantitative determination of hybrid formation.

As indicated, it is preferred to use ELISA techniques to effect or bring about the expression or indication of, qualitatively or quantitatively, of the non-radioactive chemically-labeled probe in the resulting formed double-stranded or hybridized material, such as the duplex or hybrid formed by the non-radioactive chemically-labeled DNA probe and its substantially complementary genetic DNA material derived from the denatured denetic DNA material to be identified. In the preferred ELISA technique in accordance with this invention, single-stranded genetic material, e.g. DNA, after denaturing of the DNA material to be identified to form the single-stranded DNA material, is fixed to a substrate. In the practices of this invention, it is preferred that the substrate be a transparent substrate, such as a glass substrate or surface. By employing a transparent substrate, such as a glass substrate, to which the single-stranded DNA material is fixed and hybridized, the ELISA technique provides for qualitative and quantitative determination of the DNA material to be identified. By employing a transparent substrate to which the DNA material is fixed, full realization of the benefits and versatility of the ELISA techniques applied to the practices of this invention are obtainable. Other substrates useful in practicing this invention include cellulose, nitrocellulose, and dextran, for instance in the form of a porous filter or column packing material.

It is known to fix enzymes and the like to siliceous materials and to detect antibodies and the like involving attachments of such materials as enzymes, antibodies and antigens to various substrates, such as siliceous substrates. See for example U.S. patents 3,669,841, 3,715,278, 3,949,064, 4,001,583, 4,059,685, 4,120,945 and 4,280,992. In the practices of this invention, it is highly desirable that the fixing of the denatured single-stranded genetic DNA material derived from the DNA material to be identified be rapidly fixed to the substrate, such as a transparent substrate, e.g. a glass surface. Rapid fixing of single-stranded DNA material to a transparent glass substrate would permit rapid testing of numerous samples involving ELISA techniques. For example, glass plates provided with an array of depressions or wells therein would have samples of the various denatured genetic materials to be identified deposited therein and the single-stranded DNA material therein fixed to the surfaces of the wells. Thereupon, DNA probes provided with a non-radioactive chemical label are deposited in each of the wells for hybridization to any complementary single-stranded DNA material therein. After washing to remove any non-hybridized probe, the presence of any hybrid DNA material containing the single-stranded DNA material to be identified and the non-radioactive chemically-labeled probe is detected, as described herein, involving the addition of an enzyme linked antibody or other suitable entity for attachment to the chemical label of the probe. Subsequently a suitable substrate is added to elicit a color change or chemical reaction which could then be measured colorimetrically or photometrically.

To effect rapid fixing of DNA material, such as denatured single-stranded DNA, to glass, the glass is prepared or pretreated by heating or boiling the glass, such as a borosilicate glass, for a sufficient period of time, e.g. about 45 minutes, in the presence of dilute, e.g. 5 percent,

aqueous nitric acid. This pretreatment with nitric acid serves to leach out boron residues from the glass surface. The treated glass is then washed or rinsed with water, preferably with distilled water, and dried, such as at a temperature of about 115°C, for about 24 hours. A 10 percent solution of gamma-aminopropyltriethoxysilane prepared by dissolving the above-identified silane in distilled water followed by addition of 6N hydrochloric acid to a pH of about 3.45, is then applied to the glass surface, and the glass surface is then incubated in contact with the above-identified silane solution for about 2-3 hours at a temperature of about 45°C. The glass surface is then washed with an equal volume of water and dried overnight at a temperature of about 100°C. The resulting treated glass surface has available alkylamine provided on the surface thereof suitable for immobilizing or fixing any negatively charged polyelectrolytes applied thereto. In connection with the above, see Weetal, H.H. and Filbert, A.M., "Porous Glass for Affinity Chromatography Applications", Methods in Enzymology, Vol. XXXIV, Affinity Techniques Enzyme Purification: Part B, pp. 59-72, W.B. Jakoby and M. Wilchek, eds.

To illustrate the practices of this invention, various DNA materials were prepared. For example, bacteriophage T_4 DNA was isolated from E. coli CR63 cultures infected with phage T_4 AM82 [44^-62^-] and purified to be free of chromosomal DNA. Highly purified calf thymus DNA was obtained from a biological supply service. Bacteriophage lambda was obtained by heat induction of E. coli strain W3350 lysogenic for λC_1 857 phage and was employed for the preparation of phage lambda DNA. Also, radioactive DNA was prepared by nick translation with [^3H] dATP and phage lambda DNA was also nick translated with maltose-triose dUTP to introduce glucosyl or saccharide residues into the DNA.

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Other reagents were obtained or prepared, for example, lectins which readily and eagerly form a complex with glucosyl groups. Specifically Concanavalin A [ConA] was obtained and solubilized in 2.0M NaCl at a concentration of 50 mg/ml. Also, fluorescein-labeled ConA was prepared by reacting ConA with fluorescein isothiocyanate at a FITC to protein molar ratio of 3 in 0.1M sodium borate solution at a pH of 9.2 and at a temperature of 37°C for 60 minutes. Any unreacted FITC was removed by gel filtration on Sephadex G-50 (Sephadex is a trademark).
A glass surface treated as described hereinabove was employed in the detection of lectin-binding to glucosylated DNA. In this procedure, glucosylated DNA [T_4 DNA] or non-glucosylated DNA [calf thymus DNA] was delivered in 100 µl portions to treated glass tubes in triplicate set. After 15-30 minutes at room temperature, the solution was removed and the tubes rinsed generously with PBS•Mg^{++} buffer [100mM Na-K-Po_4, pH 6.5, 150mM NaCl and 10mM $MgCl_2$]. One set of tubes was checked for the presence of DNA by staining with ethidium bromide [100 µl of 1 mg/ml solution, 30 minutes in the dark, at room temperature]. The staining solution was removed and the tubes rinsed and checked under UV light. To another set of tubes there was delivered fluorescent-labeled ConA [100 µl of 0.1 mg/ml in PBS•Mg^{++} buffer]. After 60 minutes at room temperature, the solution was removed and the tubes were rinsed and checked under UV light.

To the third set of tubes was delivered 100 µl of unlabeled ConA in PBS•Mg^{++} buffer. After 60 minutes at room temperature, the tubes were rinsed free of ConA with 0.2M Inidazole buffer pH 6.5.

Acid phosphatase was then added [0.005 units in 100 µl at 0.2 percent phosphatase-free BSA] and the tubes were incubated at room temperature for 30 minutes. After rinsing with 0.15M NaCl (to remove any unbound enzyme) substrate [para-nitrophenylphosphate 0.1mM in 0.2M Inidazole pH 6.5] was added and incubation continued for 60 minutes

at 37°C. The enzyme reaction was terminated by adding 1.0 ml of 0.5 percent sodium bicarbonate and absorbance was determined at A_{300}.

The resulting observed test results indicated that both glucosylated and non-glucosylated DNA bound to the activated glass surface by the observed red fluorescence characteristic of ethidium bromide. Also, ConA bound only to glucosylated DNA as noted by the green fluorescence in tubes containing T_4 DNA, but not in tubes which had calf thymus DNA. Further, acid phosphatase, which is a glycoprotein, gave positive reaction only in tubes containing T_4 DNA and ConA, but was washed off from the tubes which contained only ConA or ConA and calf thymus DNA.

Activated glass tubes were also employed in the detection of lectin-binding to glucosylated DNA probes hybridized to DNA already immobilized on glass surface. In these test, phage lambda DNA was immobilized on glass surface. After rinsing with buffer, the tubes were coated with 100 µl of coating solution [50 percent formamide, 5X SSC, 100 µg salmon sperm DNA, 0.2 percent polyvinyl pyrrolidone, 0.1 percent Triton* X-100, 0.2 percent BSA and 0.05 percent SDS] at 42°C for 90-120 minutes. The coating solution was removed and the surface was covered with 100 µl of coating solution containing glucosylated nick-translated DNA probe. The probe had been denatured at 80°C for 3 minutes and rapidly cooled in ice bath immediately before use. The tubes were then incubated at 42°C for 24 hours. The solution was removed and tubes were rinsed with PBS•Mg^{++} buffer. The lectin-enzyme detection system was delivered as described before. The results indicated that acid phosphatase was not washed off from the tubes which contained glucosylated DNA probe, whereas, tubes containing non-glucosylated probe did not show any enzyme activity.

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* Triton is a trademark

In these tests, the glucosyl (monosaccharide) moiety of the glucosylated DNA probe serves the non-radioactive chemical label and reacts with or is strongly attracted to the lectin, ConA, making up the combination lectin-enzyme (acid phosphatase) probe detection system.

In the above test employing glucosylated DNA as a probe, wherein the glucosyl or monosaccharide moiety of the glucosylated DNA probe serves as the non-radioactive chemical label, comparable results are also achievable in the practice of this invention employing a biotin-labeled DNA probe. When biotin is employed as the non-radioactive chemical label of the DNA probe, and since avidin is strongly reactive with or strongly bonds to biotin, the presence of the biotin-labeled DNA probe would be elicited or detected by means of avidin or streptavidin-labeled enzyme. For example, a biotin-labeled DNA probe would readily be detected by an enzyme complex of the character avidin-biotin-alkaline phosphatase. More specifically, the presence of the biotin-labeled DNA probe would readily be elicited or detected by contacting the hybrid containing the biotin-labeled probe with the enzyme complex avidin-biotin-alkaline phosphatase, followed by bringing the resulting biotin-labeled DNA probe having attached thereto the avidin-biotin-alkaline phosphatase complex into contact with a suitable substrate which by color reaction or precipitate formation brought about by the alkaline phosphatase would be readily noticed or capable of being determined, both qualitatively and quantitatively, by photometric and/or colorimetric means in accordance with ELISA techniques. If desired, instead of an avidin-biotin-enzyme complex, there could be used an antibody to biotin for attachment to the biotin moiety of the biotin-labeled DNA probe, followed by a complex comprising anti-antibody-enzyme in the manner described hereinabove.

The advantages of the practices of this invention are also obtainable when the DNA probe is fixed to or hybridized to a plastic surface, such as a polystyrene surface. When a plastic surface is employed, it is sometimes desirable, in order to increase the effectiveness or uniformity of the fixing of DNA applied thereto, including the non-radioactive chemically-labeled DNA probe, to treat the plastic surface, such as a polystyrene surface, to enhance and otherwise improve the fixing or immobilization of genetic material thereon, such as single-stranded denatured DNA or a non-radioactive chemically-labeled probe or hybrid DNA containing the DNA probe. It has been found, for example, that the adherence or fixing of DNA to a polystyrene surface is improved by treating the surface, as indicated hereinabove, with an amino-substituted hydrophobic polymer or material, such as duodecadiamine. Another technique for improving the fixing or uniformity of the plastic surface for fixing DNA involves treatment of the surface with polylysine (PPL).

In tests involving the fixing of DNA to a plastic surface, biotinylated DNA was denatured and aliquoted into Dynatech, Immulon II^{TM} removeable wells. Samples were allowed to dry onto the plastic surface at 37°C. The amount of bound b-DNA was determined by sequential addition of goat anti-biotin antibody and rabbit anti-goat antibody complexed to alkaline phosphatase, followed by development with p-nitrophenyl phosphate in diethanolamine buffer, pH 9.6. Enzymatic activity was monitored at 405 nm utilizing the automatic Dynatech Micro ELISA Scanner. This procedure enables the investigator to quantitate the amount of bound DNA and circumspectively the degree of biotinylation.

To increase the sensitivity of detection, a fluorogenic substrate, such as 4-methylumbelliferyl-phosphate, or its analogues, with companion enzymes, may be used.

Additional tests were carried out wherein several aliquots of denatured adenovirus 2 DNA were bound to polystyrene plates as described above. After blocking with Denhardt's formamide blocking buffer, several biotinylated probes were hybridized to the immobilized DNA; these were B-adeno-2-DNA and lambda DNA. To one set of immobilized DNA, no probe was added. The extent of hybridization was determined by means of the antibody-enzyme reaction as described above. It was observed that only the homologous adeno-2 probe hybridized. This technique demonstrated that _in vitro_ hybridization under these conditions is specific and can be monitored quantitatively.

Polystyrene from various batches or sources exhibits different binding capacities. Previous experiments had demonstrated that addition of duodecadiamine (DDA) to polystyrene resulted in an uniform binding coefficient of polystyrene plates of different batches.

In further tests, radioactively-labeled, non-biotinylated denatured DNA [2000 ng to 5 ng] was applied to DDA-coated polystyrene plates. The test samples or plates were not allowed to dry. After incubation at 37°C for 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 18 hours, samples were counted. Binding was maximal after 2 hours of incubation, however, 50 percent of the originally applied DNA bound regardless of the concentration, thereby indicating that there is an equilibrium between bound and unbound DNA.

In other tests, polystyrene microfilter wells were nitrated using a procedure of Filipsson and Hornby [Biochem J. 120 215 (1970)]. The polystyrene wells were immersed for 20 minutes in a mixture of concentrated nitric and sulfuric acid [41 percent, v/v] cooled to 0°C. The wells were then washed thoroughly with water and subsequently heated to 70°C in a 6 percent solution of sodium dithionate in 2M potassium hydroxide. After 4 hours, the wells were washed thoroughly with 0.5M hydrochloric acid and distilled water.

To produce 6-aminohexane linked polystyrene, 6-aminocaproic acid-N-hydroxysuccinimide ester•hydrobromide [5 mg thereof dissolved in 0.2M dimethylformamide prepared by reacting 6-aminocaproic acid•hydrobromide with N-hydroxysuccinimide and dicyclohexyl carbodiimide in dimethylformamide and recrystallized from isopropylalcohol] was added to 0.1M sodium borate [0.4 ml]. Amino-derivitized polystyrene microfilter wells filled with this solution were allowed to react at room temperature for 4 hours and then washed thoroughly with distilled water. The resulting treated wells absorbed ^{3}H-labeled DNA from aqueous solution at pH less than 9.5.

As indicated hereinabove, siliceous substrates, such as glass substrates, and plastic substrates, such as polystyrene substrates, are improved with respect to the capability of fixing or immobilization of DNA thereto by treatment with or by providing thereon a coating of an epoxy resin. For example, glass and polystyrene surfaces are improved with respect to the fixing or immobilization of DNA thereon by treatment of glass or polystyrene surfaces with commercially available epoxy glues, such as a solution of epoxy glue in ethanol [1 percent w/v]. These epoxy solutions are applied to the glass, e.g. Pyrex*, glass surfaces or polystyrene surfaces or wells, and the solvent, ethanol, evaporated thereupon at a temperature of 37°C,

* Pyrex is a trademark.

thereby providing a polyamine polymeric coating on the thus-treated surface. These surfaces were found to absorb ^{3}H-labeled DNA from aqueous solution at pH less than 9.5.

The methods described hereinabove with respect to ELISA-like techniques for the colorimetric or photometric determination of the hybridized probes involve enzyme-linked reagents which produce a color change in a substrate or a precipitate. Various combinations of enzymes and color-producing substrates may be employed. See for example accompanying tables, Table I and Table II, showing the combination of enzymes and chromogens. In Table I, the chromogen found in the substrate is reactive with the enzyme employed to elicit or determine the presence of the non-radioactive chemically-labeled probe. The superscript notation (F) indicates that the chromogen fluoresces. In accompanying Table II, there are set forth those chromogens which produce an insoluble product, such as when employed with the avidin/streptavidin system. These combinations of enzyme-chromogen are particularly useful in ELISA techniques for the determination, both qualitatively and quantitatively, of the hybrid DNA or other genetic material containing the special non-radioactive chemically-labeled probe in accordance with this invention.

TABLE I

ENZYME	CHROMOGEN
1a. alkaline phosphatase	4-Methylumbelliferyl phosphate[(F)]
b. acid phosphatase	bis (4-Methylumbelliferyl phosphate)[(F)]
	3-0-methylfluorescein.
	Flavone-3-diphosphate triammonium[(F)] salt
	p-nitrophenyl phosphate 2Na.
2. peroxidase	Tyramine hydrochloride[(F)]
	3-(p-hydroxyphenyl) Propionic acid[(F)]
	p-Hydroxyphenethyl alcohol[(F)]
	2,2'-Azino-Di-3-Ethylbenzthiazoline sulfonic acid (ABTS)
	ortho-phenylenediamine 2HCl
	o-dianisidine
	5-aminosalicylic acid
	p-cresol[(F)]
	3,3'-dimethyloxybenzidine
	3-methyl-2-benzothiazoline hydrazone
	tetramethyl benzidine
3. β-D-galactosidase	0-nitrophenyl β-D-galactopyranoside
	4-methylumbelliferyl-β-D-galactoside
4. glucose-oxidase	ABTS

T A B L E I I

CHROMOGEN (insoluble product)

ENZYME	
1. alkaline phosphatase	6-bromo-3-hydroxy-2-naphthyl-0 anisidine + Fast Violet B salt.
	2,2'-dimethyl-3-hydroxy-2-naphthanilide + Fast Red salt.
	flavone-3-diphosphate ammonium salt + $AlCl_3$ (F)
	5-bromo-4-chloro-3-indolylphosphate + tetrazolium, nitro BT.
2. Horseradish peroxidase	H_2O_2 + diaminobenzidine
	H_2O_2 + tetramethylbenzidine
3. Glucose oxidase	glucose + thiazolyl blue
4. β-galactosidase	2-(β-D-galactosidoxy) naphthol AS-LC + Fast Violet B salt.
	Naphthol-AS-Bl-B-0-galactopyranoside + Fast Blue BBN salt.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations, modifications and substitutions are possible in the practice of this invention, without departing from the spirit or scope thereof.